Soluble N-Ethylmaleimide-sensitive Fusion Attachment Proteins (SNAPs) Bind to a Multi-SNAP Receptor Complex in Golgi Membranes*

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Soluble N-ethylmaleimide-sensitive fusion attachment proteins (SNAPs) are required for the binding of N-ethylmaleimide-sensitive fusion protein (NSF) to Golgi membranes and are, therefore, required for intra-Golgi transport. We report the existence of distinct α/β-SNAP and γ-SNAP-binding sites in Golgi membranes that appear to be part of the same receptor complex. Cross-linking studies with α-SNAP demonstrate that an integral membrane protein of between 30–40 kDa is the α-SNAP binding component of the multi-SNAP receptor complex. These data suggest that SNAPs function by independently binding to a multi-SNAP membrane-receptor complex, thereby activating them to serve as adaptors for the targeting of NSF.

Vectorial transport of proteins via non-clathrin-coated vesicles is an essential cellular process and must be tightly controlled to assure the correct distribution of cellular components. In vitro reconstitution of intercisternal Golgi transport (Rothman and Orci, 1991) has led to the discovery and purification of several proteins that are involved at various stages of intra-Golgi transport. The first of these proteins, NEM*-sensitive fusion protein, (NSF) (Block et al., 1988) is required after transfer and uncoating of non-clathrin-coated transport vesicles and thus appears to be necessary for fusion of the vesicle and acceptor membrane bilayers (Malhotra et al., 1988). NSF is a tetrameric protein of identical 76-kDa subunits (Block et al., 1988) that has two consensus ATP-binding sites/subunit (Wilson et al., 1989).

A second family of proteins, termed soluble NSF attachment proteins (SNAPs), is also required at a late stage in transport and is essential for the specific interaction of NSF with membranes (Weidman et al., 1989; Clary et al., 1990). Three species of SNAPs have been identified: α-SNAP (35 kDa), β-SNAP (36 kDa), and γ-SNAP (39 kDa) (Clary and Rothman, 1990). While each of the SNAPs is sufficient to promote intercisternal Golgi transport, they differ in their specific activities (α>β>γ, (Clary et al., 1990)).

An important question raised by this is whether each of the SNAPs plays a distinct role in the targeting and fusion process. To address this question, we have studied the interactions of the SNAP proteins with NSF and Golgi membranes. Initially, it was reported that the SNAP activity and an undefined integral Golgi membrane protein were responsible for the specific interaction of NSF and membranes (Weidman et al., 1989). In the further investigation of this process, we find that SNAPs do not interact with NSF in solution, but specifically bind to a membrane receptor and, thus, target NSF to the membrane. Additionally, α- and γ-SNAP bind to distinct receptor sites that are part of the same complex. A combination of α- and γ-SNAP provides the optimal condition for NSF binding to membranes.

EXPERIMENTAL PROCEDURES

Preparation of Unlabeled SNAPs and [35S]α-SNAP—Purification of SNAPs was performed according to the method of Clary and Rothman (Clary and Rothman, 1990). The cloning of the α-SNAP cDNA will be described elsewhere1 and was accomplished by conventional techniques from a bovine brain cDNA library. In vitro transcription and translation of α-SNAP was done by conventional techniques using T3 RNA polymerase and rabbit reticulocyte lysate (Promega, Madison, WI) with [35S]methionine (Amersham Corp.) (Krieg and Melton, 1987). The subsequent enrichment of the [35S]-α-SNAP was done by ammonium sulfate precipitation using a 30–50% cut as described (Clary and Rothman, 1990). The functional assay of α-SNAP generated in vitro, (over endogenous SNAPs in reticulocyte lysate) using 1 mM methionine, was done with KCl-extracted Golgi membranes according to (Clary and Rothman, 1990). Specific activity measurements for [35S]α-SNAP were done with anti-peptide antibodies generated against a synthetic peptide from α-SNAP. This peptide (H-Y-E-Q-S-A-D-Y-Y-K-G-E-E-C) was coupled to Immune Acted Super Carrier (Pierce Chemical Co.) and was used to elicit polyclonal antibodies in rabbits by East Acres Biologicals (Southbridge, MA); the resulting antibodies were affinity purified using peptide coupled to Sulfolink resin (Pierce). The specific activity of the radio-labeled α-SNAP was determined by Western blotting using a standard curve of known protein concentration to determine the amount of protein made and the radio-labeled α-SNAP was measured by liquid scintillation counting.

Preparation of Rat Liver Golgi Membranes—Rat liver Golgi membranes were prepared by standard procedures as reported (Malhotra et al., 1989) using frozen rat liver (PelFreeze, Rogers, AK) as a source of starting material. The salt stripping was performed according to Clary et al. (1990). Proteinase treatment of membranes was done at 4°C and 23°C for 60 min and at 37°C for 20 min with Proteinase K (1 mg/ml final). The proteolysis reactions were stopped by adding phenylmethylsulfonyl fluoride (5 mM final, from a 100 mM stock in 2 M. Brunner, S. W. Whiteheart, and J. E. Rothman, manuscript in preparation.)
isopropyl alcohol). The membranes were harvested by sedimentation and resuspended in their original volume of 30% sucrose 10 mM Tris-HCl pH 7.4, 1 mM phenylmethylsulfonyl fluoride. The detergent-
solubilized membrane fraction used in the experiment represented by Fig. 1 was prepared by salt-extracting membrane from bovine brain with 1 mM KCl. The salt-extracted membranes were solubilized in 1% Triton X-100 and dialyzed against 25 mM Tris-HCl, pH 7.8, 50 mM KCl, 1 mM dithiothreitol, and 1% Triton X-100.

**Plastic Binding Assay of SNAP-NSF Interactions**—SNAP (20 ng) was preadsorbed to 1.5-ml microfuge tubes in 25 mM Tris-HCl, pH 7.8, 50 mM KCl, and 1 mM dithiothreitol for 20 min on ice. The tubes were then blocked for 20 min by the addition of 200 μl of 10 mg/ml bovine serum albumin, NSF (25 ng) was added, and binding assays were carried out according to Clary et al. (1990). In order to measure competition, soluble SNAPs were added during the NSF-binding step. When no SNAPs were preadsorbed, there was a background binding of 1243 counts/min.

α-SNAP Binding Assays—Binding experiments were done in NB buffer (20 mM HEPES, KOH, pH 7.4, 100 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, 0.25 mM ATP, 1% PEG-4000, and 0.25 mg/ml soybean trypsin inhibitor (Clary et al., 1990)) in a final volume of 50 μl. After incubation for 5 min at 4 °C (or 30 °C), the SNAP bound to membranes was harvested by centrifugation through a 25% sucrose in NB buffer cushion (120 μl) at 9000 rpm for 30 min in an Optima TL, ultracentrifuge (TLA-100.1 rotor, Beckman, Palo Alto, CA). The pelleted membranes were suspended and the bound [35S]α-SNAP was quantitated by liquid scintillation counting. When membranes were tittered into the binding assay, a maximum of 28% of the added [35S]α-SNAP could be bound.

γ-SNAP Binding Assays—Binding experiments were done in NB buffer in a final volume of 75 μl. After incubation for 30 min at 30 °C, the γ-SNP bound to membranes was harvested by centrifugation (14,000 X g, 7 min, 4 °C). The membrane pellet was solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (Laemmli, 1970), and the γ-SNP binding was quantitated by Western blot with anti-peptide antibodies generated using a peptide sequence determined from chemical sequencing of γ-SNP (L-E-R-A-N-V-D-P-E-K-C). The secondary antibody was a goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad), and chromophore was generated using 5-bromo-4-chloro-3-indoly phosphate and Nitro Blue tetrazolium in 100 mM Tris-HCl, pH 8.8, 1 mM MgCl₂. The Western blots were scanned with a Scan Jet Plus scanner (Hewlett Packard, CA), and the images were integrated using the Scan Analysis software from BioSoft (Cambridge, United Kingdom).

Preparation of α-SNP-Sepharose—α-SNAP was expressed with six consecutive histidines at the N terminus to aid in purification (Qiagen Products). The protein was purified as described by Brunner et al. [α-SNP-Sepharose was made by coupling α-SNP-His₆ to CNBr-activated Sepharose 6MB from Sigma following manufacturer’s instructions at a coupling density of 10 mg of α-SNP/g of resin. Cross-linking of [35S]α-SNAP to Golgi Membrane Proteins—[35S]α-SNAP was bound to salt-extracted rat liver Golgi membranes as in Fig. 1, the membranes were harvested by centrifugation as above, and the γ-SNP bound in 50 μl of 25 mM HEPES-KOH, pH 7.4, 100 mM KCl. DSS was added from a stock (2.5 mg/ml in 50% dimethyl sulfoxide) to a final concentration of 0.25 mg/ml and incubated on ice for 20 min. The reaction was quenched with 100 mM glycy melanchine, pH 7.4, for 30 min on ice, and the membranes were washed for 10 times with 3 ml of a solution composed of 100 mM NaOAc, pH 11.5. NSF used in these experiments was a bacterially expressed NSF construct with an epitope on whether SNAPs could bind to membranes in the absence of NSF. In each case, the same type of SNAP adsorbed was used as competitor.

The total NSF activity added to the binding assay was 3711 cpm as measured by the NSF-dependent transport of VSV-G protein and its subsequent glycosylation by N-acetylglucosaminyl transferase I and UDP-[5H]N-acetylglucosamine (Block et al., 1988). SNAPs do not interact in solution, we took advantage of a solid-phase assay in which one or more SNAPs are first adsorbed to a plastic surface and then exposed to NSF. Under these conditions, each of the three SNAPs can mediate the binding of NSF to the plastic surface (Clary et al., 1990). However, when free SNAPs were included at a concentration of 25-fold excess over monomer NSF (which is 10-fold excess over plastic bound SNAPs), they could not compete with the immobilized, plastic-bound SNAPs for binding to NSF (Table I). This suggests that when a SNAP is adsorbed to a hydrophobic surface, it gains the ability to bind NSF. It is possible that the plastic surface in some way mimics the action of a membrane receptor inducing a conformational change in the SNAPs which enables them to bind NSF.

α-SNAP Binds to Golgi Membranes—Since NSF and SNAPs do not appear to interact in solution, and since NSF does not bind to membranes in the absence of SNAPs (Clary et al., 1990; Weidman et al., 1989) we focused our attention on whether SNAPs could bind to membranes in the absence of NSF and thus serve as a pool of adaptors for NSF binding. This seemed to be likely since, as purified, the SNAPs are peripheral membrane proteins (Clary and Rothman, 1990), and an integral membrane protein has been reported to be involved in NSF binding (Weidman et al., 1989). We employed in vitro translated [35S]α-SNP, which was demonstrated to be active in a SNAP-dependent transport assay (data not shown), to show specific binding of α-SNP to Golgi membranes (Fig. 1, A and B). The [35S]α-SNP binding in a saturable fashion with a Kₘ of 14 ± 5 nM as estimated from linear Scatchard plots of three separate binding experiments. The number of binding sites calculated from this analysis was 38 ± 15 pmol/ml of salt-washed rat liver Golgi membranes, which is similar to the number of binding sites reported for NSF (50 pmol/ml (Weidman et al., 1989)). Multimellar and unimellar phosphatidylcholine liposomes showed no binding activity. Proteinase K treatment of salt-washed Golgi at 4 °C, 23 °C, or 37 °C (1 mg/ml Proteinase K, 20 min) or 37 °C (1 mg/ml Proteinase K, 20 min) reduced α-SNP binding by 73, 71, or 99%, respectively (Fig. 1C). The protease digestion conditions previously reported (0.1 mg/ml, 2 h, on ice (Weidman et al., 1989)) were not stringent enough and did not affect the binding of α-SNP to Golgi membranes. Inclusion of ATP (0.25 mM) or Mg₂⁺/ATP (2 mM, 0.25 mM) were without effect on α-SNP binding (data not shown).

Unlabeled α-SNP from bovine brain competed with the labeled α-SNP for binding as did β-SNP, however, γ-SNP did not compete for binding (Fig. 1D). The half-maximal concentration for this inhibition by both α- and β-

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**NSF and SNAP Interactions**

**RESULTS**

**NSF and SNAPs Do Not Interact in Solution**—We were unable to detect any interaction between NSF and any of the three SNAP proteins, alone or in combination. Stable complexes of SNAPs and NSF could not be isolated by glycerol gradient centrifugation or gel filtration chromatography (data not shown) and SNAPs did not stimulate the ATPase activity of NSF. To demonstrate in a positive way that NSF and

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SNAP was about 20 nM, similar to the binding constant calculated for α-SNAP. The similar binding of α- and β-SNAP is, perhaps, related to their extensive sequence homology; γ-SNAP, by comparison, shows no sequence homology to α-SNAP. α-SNAP binds to Golgi membranes equally well at both 4 and 30 °C (Fig. 1E).

As is shown in Fig. 1E, γ-SNAP or NSF alone or in combination have no significant effect on α-SNAP binding, indicating that α-SNAP binding is independent of these other transport components. Notably, NSF binding to membranes (determined by Western blotting and densitometry, see Fig. 2C) was 2.7-fold better when both α- and γ-SNAP were present than when either alone was present.

γ-SNAP Binds to Golgi Membranes—Since an active radiolabeled source of γ-SNAP is not yet available binding of γ-SNAP to membranes was quantitated by Western blotting and densitometry using purified γ-SNAP and an anti-γ-SNAP-peptide antibody specific for γ-SNAP (Fig. 2A). Binding of γ-SNAP was easily detected when incubations were done at 30 °C, but binding was 3-fold lower at 4 °C. Protease treatment of the membranes reduced γ-SNAP binding by 34, 46, or 63% when the membranes were treated at 4, 23, or 37 °C, respectively (Fig. 2B). γ-SNAP binding was not competed for by α-SNAP, but, instead, was slightly stimulated (Fig. 2C). Addition of NSF reduces the binding of γ-SNAP (Fig. 2C). As seen for α-SNAP binding, inclusion of ATP or Mg²⁺/ATP did not significantly affect γ-SNAP binding to membranes (data not shown). Using these techniques, we were unable to demonstrate saturability of γ-SNAP binding in Fig. 2A mainly because it was not possible to add enough of our purified protein to the binding assay. In order to produce a consistent signal in the binding assay, it was necessary to use larger amounts of membranes which in turn required more γ-SNAP to saturate than we were able to add. However, we believe that the binding of γ-SNAP is specific to a receptor because it is reduced by protease treatment of the membranes and the binding is affected by α-SNAP and NSF. While γ-SNAP binding needs to be studied with more quantitative methods, it is clear that γ-SNAP binds to a membrane receptor protein at a site that is distinct from the α/β-SNAP-binding site. As both α/β- and γ-SNAP binding remain after carbonate extraction of the membranes (data not shown), it is likely that both binding sites exist on integral membrane protein(s).

The α-SNAP and γ-SNAP-binding Sites Are Part of the Same Complex—To investigate whether the α-SNAP- and γ-SNAP-binding sites are part of the same complex, α-SNAP was coupled to Sepharose 6B to make an affinity resin. The rationale is that if the γ-SNAP-binding site is part of the same complex as the α-SNAP site then when the α-SNAP receptor binds to the affinity resin, γ-SNAP will also bind and associate with the Sepharose beads. As shown in Fig. 3, γ-SNAP is only seen associated with the α-SNAP-Sepharose when a detergent-solubilized (i.e. α-SNAP receptor containing) membrane fraction is present in the incubation. These data show that the distinct α/β- and γ-SNAP-binding sites are part of the same membrane complex but do not reveal

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whether these sites exist in the same polypeptide chain, or in two associated chains. This of course, awaits purification of the multi-SNAP receptor.

Cross-linking of \[^{35}S\]a-SNAP to Golgi Membrane Proteins—In an effort to identify proteins which are proximal to specifically bound \(\alpha\)-SNAP on the membrane, \(\i.e\). to identify candidates for binding components of the multi-SNAP receptor, we undertook cross-linking studies. Several cross-linking reagents (CMC, Sulfo-MBS, and BS\(^3\)) were tested, and it was determined that DSS was most effective at generating intermolecular cross-linked products when used at the concentration of 0.25 mg/ml. Fig. 4A shows the results of an experiment in which all of the samples were subjected to immunoprecipitation with an anti-\(\alpha\)-SNAP antibody after cross-linking. Lanes 3 and 6 demonstrate that the cross-linked products are \(\alpha\)-SNAP specific in that unlabeled \(\alpha\)-SNAP competes with their formation. The labeled band at about 116 kDa in lanes 5 and 7 represents the cross-linked product of \(\alpha\)-SNAP and NSF; it is only seen when NSF is present in the reaction, is peripheraly associated with the membranes because it is \(\text{CO}_{2}^-\)-extractable (Fig. 4B), and is immunoprecipitable with an anti-SNAP antibody (data not shown; see "Experimental Procedures"). This confirms that \(\alpha\)-SNAP is in physical contact with NSF in the membrane-bound complex. These same patterns of cross-linked products were seen when a \[^{35}S\]a-SNAP which had N-4-(3-trifluoromethyl)diazirino)benzoyllysine incorporated into the polypeptide during translation (Gorlich et al., 1991) was used for photoactivated cross-linking experiments (data not shown). Fig. 4B shows the results of an experiment in which the membranes were extracted with \(\text{CO}_{2}^-\) after cross-linking but before gel electrophoresis. The labeled band at about 74 kDa is the only cross-linked product that is carbonate-extraction resistant suggesting that it involves an integral membrane protein. Subtracting the molecular mass of \(\alpha\)-SNAP (35 kDa), this would mean that this integral membrane protein has an approximate molecular mass of 30-40 kDa. It is unlikely that the 74-kDa cross-linked product represents an \(\alpha\)-\(\alpha\)-SNAP or \(\alpha\)-\(\gamma\)-SNAP cross-link since both \(\alpha\) and \(\gamma\)-SNAP are completely removed by \(\text{CO}_{2}^-\) extraction as demonstrated by Western blotting of extracted membrane proteins with anti-SNAP antibodies (data not shown). The \(\text{CO}_{2}^-\) extractable cross-link products appear to represent other peripheral membrane proteins that are in association with \(\alpha\)-SNAP.

**DISCUSSION**

These data clearly demonstrate the existence of an \(\alpha\)-SNAP receptor, which can also bind \(\beta\)-SNAP, and a distinct \(\gamma\)-SNAP receptor. The SNAP-binding sites are integral membrane protein components which are either on the same polypeptide chain or on separate proteins in a tight complex. \(\alpha\)-SNAP binding is independent of \(\gamma\)-SNAP, NSF, and Mg\(^{++}\)/ATP. The binding of \(\gamma\)-SNAP is positively affected by \(\alpha\)-SNAP, negatively affected by NSF, but is not affected by
Mg\textsuperscript{2+}/ATP. Chemical and photoactivatable cross-linking studies have identified one integral membrane component and several peripheral membrane components which appear to be part of the SNAP receptor complex.

The cross-linking experiments clearly identify one integral membrane protein candidate for the α-SNAP receptor, which we are currently attempting to purify. Additionally, several peripheral membrane proteins are in close contact to bound α-SNAP. Although these are not necessary for α-SNAP binding, since α-SNAP will bind to CO\textsuperscript{2+}-extracted membranes, they may play a role in the function of bound SNAP. The presence of a NSF-α-SNAP cross-linked product is the first direct demonstration that NSF and α-SNAP physically interact when bound to Golgi membranes. This interaction was first suggested by Weidman et al. (1989) and further supported by the fact that certain alleles of the yeast homologues of NSF (Sec18p (Wilson et al., 1989)) and α-SNAP (Sec17p (Clary et al., 1990)), when combined, show synthetic lethality (Kaiser and Schekman, 1990). Since this interaction does not occur in solution, we suggest that α-SNAP, once bound to its receptor, undergoes a conformational change which makes it competent to interact with NSF.

NSF and SNAPs have been shown to act late in the transport process; after the non-clathrin-coated transport vesicles have uncoated but before they have fused with their target Golgi cisternae (Malhotra et al., 1988; Clary et al., 1990). This indicates that the correct positioning of NSF is a critical step in mediating the fusion of transport vesicles and Golgi cisternae. The data presented suggest that the initial event in the targeting of NSF is the binding of SNAPs to the SNAP receptor complex. While one SNAP (α, β, or γ) is sufficient (Clary et al., 1990), when both binding sites (α and γ sites) are filled, NSF binds and forms a more stable NSF-SNAP-receptor complex which is poised to mediate the fusion event. The SNAPs, in this way, serve as regulatory subunits that, when bound to their receptors, direct the binding of NSF to the correct site on the Golgi membranes.

Formation of the NSF-SNAP-receptor complex occurs in the presence of EDTA, which limits ATP hydrolysis, but when excess Mg\textsuperscript{2+} is added to facilitate ATP hydrolysis, NSF is released from the membranes (Weidman et al., 1989). Since Mg\textsuperscript{2+}/ATP does not affect the binding of the SNAPs to their receptor, it would appear that the effect of Mg\textsuperscript{2+}/ATP is on the NSF-SNAP interaction. NSF has a weak intrinsic ATPase activity (data not shown) which could serve to regulate the disassembly of the NSF-SNAP-receptor complex.

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